1-1-2010

The two-faced nature of small heat shock proteins: amyloid assembly and the inhibition of fibril formation. Relevance to disease states

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Abstract

The ability of small heat-shock proteins (sHsps) such as alphaB-crystallin to inhibit the amorphous (disordered) aggregation of varied target proteins in a chaperone-like manner has been well described. The mechanistic details of this action are not understood. Amyloid fibril formation is an alternative off-folding pathway that leads to highly ordered beta-sheet-containing aggregates. Amyloid fibril formation is associated with a broad range of protein conformational diseases such as Alzhiemer's, Parkinson's and Huntington's and sHsp expression is elevated in the protein deposits that are characteristic of these disease states. The ability of sHsps to prevent fibril formation has been less well characterised. It has been shown, however, that sHsps are potent inhibitors of fibril formation of a range of target proteins. In this chapter, the disease-related significance of this observation is discussed. Interestingly, in addition to being effective molecular chaperones, alphaA- and alpaB-crystallin themselves, along with some of their peptide fragments, readily form amyloid fibrils under slightly destabilising solution conditions. The implications of this observation in terms of protein conformational diseases, e.g. cataract, along with the potential nanotechnological applications of these fibrils, are discussed.

Keywords

two, faced, nature, small, heat, shock, proteins, Amyloid, assembly, inhibition, fibril, formation, Relevance, disease, states, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

Publication Details

Ecroyd, H., Meehan, S. & Carver, J. A. (2010). The two-faced nature of small heat shock proteins: amyloid assembly and the inhibition of fibril formation. Relevance to disease states. In S. Labo & A. Arrigo (Eds.), Small stress proteins and human diseases (pp. 189-211). New York: Nova Science Publishers.

The two-faced nature of small heat-shock proteins: amyloid fibril assembly and the inhibition of fibril formation. Relevance to disease states.

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1. Protein folding, misfolding and aggregation: the role of molecular chaperones

Globular proteins do not exist as structured, folded entities for their entire life. For example, after synthesis, the polypeptide chain is unstructured and must fold to its correct three-dimensional, functional conformation. To cross a biological membrane or move from one cellular compartment to another, a protein must unfold from its native state. Furthermore, if a protein is subject to stress, e.g. elevated temperature, low pH, oxidative conditions etc., it may partially unfold to adopt intermediately folded states. Many of these intermediately folded states have characteristics of molten globule states, i.e. they have elements of secondary structure but little or no tertiary structure, and are dynamic species with an expanded structure relative to the native state and have their hydrophobic core exposed to solution. The greater exposure of hydrophobicity to solution encourages protein misfolding, mutual association (aggregation) and potentially, precipitation. The cell has various mechanisms available to counteract these processes of which arguably the most important is the utilisation of molecular chaperones, a large group of structurally unrelated proteins which interact with destabilised proteins to prevent their aggregation and facilitate their correct fold [1]. Expression of molecular chaperones is increased under stress conditions, e.g. elevated temperature. Hence, they are often known as heat-shock proteins (Hsps). Thus, for a newly synthesised protein in the crowded environment of the cell, ATP-dependent molecular chaperones such as Hsp70 and Hsp60 ensure high fidelity of protein folding and assembly [1]. In this process, Hsp70 interacts initially with the unfolded, nascent polypeptide chain, specifically with extended regions of the chain. If the protein requires further encouragement to fold correctly, it is sequestered into the large cavity of the Hsp60 aggregate, where it can be isolated

from the surrounding cellular milieu, and fold via repeated interactions with the wall of the Hsp60 cavity [1]. If a protein is irretrievably misfolded and therefore incapable of folding properly, e.g. as a result of mutation, it may be labelled with ubiquitin and thereby targeted for degradation into its constituent amino acids via the proteasome, a large multi-subunit ordered complex with a large central cavity into with the targeted protein is inserted and digested.

The folding pathway for a protein proceeds via a series of intermediately folded states and is relatively rapid (Figure 1). Most newly synthesised proteins fold in a few milliseconds from their unstructured state so that the intermediates are only transiently present. However, if they linger for too long, e.g. because of difficulty in folding, mutation or due to cellular stress, these states may associate and enter the slow off-folding pathways which, if not recognised by the ubiquitin/proteasome system, eventually lead to precipitated amorphous or amyloid fibril aggregates (Figure 1). In many cases, aggregation and precipitation of proteins are highly deleterious to cell viability and are hallmarks of protein misfolding diseases (more below). In all these diseases, with the possible exception of cataract [2], the precipitated state of the proteins is highly ordered, existing in a cross β-sheet array arranged into long amyloid fibrils (more below) [3-5]. By contrast, as the name implies, the amorphous aggregation pathway is composed of cellular aggregates that are relatively disordered in arrangement. Both pathways arise from association of intermediately folded states of proteins via a nucleation-dependent mechanism (Figure 1). In the case of the amorphous aggregation pathway, this leads to the formation of aggregates that when a critical size (a nucleus) is reached, readily sequester other intermediates and form large aggregates that are intrinsically unstable and precipitate [6, 7].

Intracellularly, a unique class of ubiquitous and abundant ATP-independent molecular chaperones, the small heat-shock proteins (sHsps), interacts specifically with partially folded proteins that enter the off-folding protein aggregation pathways (Figure 2). The ability of sHsps to prevent amorphous protein aggregation was described over 15 years ago [8] and led to major research activity in relation to the principal lens protein α-crystallin, a sHsp, and its involvement in the prevention of protein aggregation in the lens and hence cataract formation [6]. A mechanism for this action has been proposed (see Figure 2 for a description) [6, 9], although specific details, at the atomic level, about the mechanism of sHsp chaperone action are not known.

2. Amyloid fibrils: mechanism of formation, structure and their association with disease.

The reasons for a protein entering either the amorphous or amyloid fibril-forming, off-folding pathways are not entirely understood but may involve one or more of the following factors:

1. The amino acid sequence of the protein: some sequences are more prone to fibril formation since the physiochemical properties of the amino acids are highly influential on fibril-forming propensity of

a polypeptide sequence [10-12]. For example, factors such as charge, hydrophobicity and propensity to adopt a β-sheet configuration are likely to play important roles. There are a variety of algorithms available to predict the fibril-forming propensity of amino acid sequences [12-15].

2. The kinetics of aggregation, with a slow aggregation rate favouring ordered, amyloid fibril formation.

3. The 'nature' of the intermediate state from which aggregation occurs, i.e. what elements of secondary structure (e.g. β-sheet in the case of amyloid fibril formation) are in place within the intermediate to facilitate protein association and aggregation along either pathway.

The misfolding, and subsequent formation of amyloid fibrils, of a normally soluble protein or peptide precursor, is associated with a variety of debilitating diseases, including Alzheimer's disease (AD), Parkinson's disease, Creutzfeldt-Jakob and Mad-Cow disease, Huntington's disease and type II-diabetes [5, 7, 16-18]. The incidence of many of these diseases will grow over the next 20-30 years as the population ages. In each case, the proteinaceous deposits or plaques that are hallmarks of the disease predominately consist of one protein or peptide, for example amyloid-β peptides (Aβ) in AD, α-synuclein in Parkinson's, prion protein in Creutzfeldt-Jakob, huntingtin in Huntington's and amylin in type II-diabetes. The severity of the disease, however, does not necessarily correlate with the extent of amyloid deposition [16], and precursors to amyloid fibrils, or small oligomeric species in particular, are believed to be especially toxic to cells [19, 20], although, the mature fibril can also be toxic [21, 22], and the most cytotoxic species may vary depending on the fibril-forming protein.

Amyloid fibrils are long thread-like protein aggregates typically 2-10 nm in diameter and ranging from one to many microns in length, as visualized by transmission electron microscopy (TEM) [23] and atomic force microscopy (AFM) [24, 25] (Figure 3). Amyloid fibrils are characterized by a 'cross-β' X-ray fibre diffraction pattern arising from a predominantly β-sheet structure, comprising two major anisotropic reflections: a meridional reflection at 4.7 Å, corresponding to the distance between β-strands, and an equatorial reflection, ca. 9-11 Å, corresponding to the distance between β-sheets [26, 27] (Figure 3). The anisotropy arises since the two aforementioned distances are perpendicular to each other; the β-strands align to form β-sheets, stabilized by extensive hydrogen bonding that run throughout the length of the fibrils, and these β-sheets stack to form protofilaments, which in turn assemble in a helical fashion to comprise a 'mature' amyloid fibril [28-30]. Fibrils exhibit a positive interaction with the dyes Congo red and thioflavin T (ThT) and hence these dyes are frequently used to monitor amyloid fibril assembly [31, 32]. It has been proposed that intercalation of the dye molecules into the extended β-sheet structure of the fibrils gives rise to either a characteristic spectral spectral shift for Congo red or a change in the fluorescence properties/emission intensities of ThT [33]. A

range of biophysical techniques (i.e. TEM, AFM, X-ray fibre diffraction, and dye binding) are useful in characterizing amyloid fibril assembly.

The ability to assemble into amyloid fibrils has been proposed to be a generic property of polypeptide chains [4], although the propensity of proteins to aggregate to form fibrils can vary widely depending on the factors listed above and solution conditions [34]. The amyloid pathway proceeds via a soluble protofibril precursor (nucleus) that acts as a template to sequester other intermediates, via a nucleation-dependent mechanism, which eventually leads to the insoluble amyloid fibril [5, 34]. This mechanism is consistent with the observed kinetics of fibril formation, as monitored using ThT or Congo red. Thus, both the length of the lag phase (i.e. the time taken to form a stable nucleus) and the rate of elongation are highly dependent on the protein concentration through their reliance on the concentration of partially folded intermediates present at any given time [35]. Whilst this nucleation-dependent mechanism holds for most *in vitro* amyloid fibril forming species studied to date, alternative mechanisms do exist [36].

3. Amyloid fibril formation by α-crystallin: *in vitro* studies.

Although renowned for their impressive chaperone ability, i.e. the capability to prevent target proteins from aggregating amorphously or to form amyloid fibrils [37], sHsps themselves can self-assemble into amyloid fibrils [38-41]. α-Crystallin is the principal lens protein and is comprised of two closely related subunits, αA- and αB-crystallin. Amyloid fibril formation is observed for a variety of α-crystallin isoforms *in vitro* (Figure 4), as summarized by Table 1, which lists the protein source and the relevant references describing their fibril formation. For example, both wild-type and mutant human recombinant αA- and αB-crystallins form aggregates displaying the characteristic properties of amyloid fibrils, including long filamentous structures as observed by TEM (Figure 4B and C), a positive interaction with Congo red, and cross-β X-ray fibre patterns [39].

Amyloid fibrils can be produced from α-crystallins obtained from a variety of sources, including bovine (Figure 4A) [38], deer, and sheep eye lenses [41]. Both purified α-crystallin fractions and crude protein extracts, containing a mixture of crystallin proteins together with other unrelated minor crystallins (β and γ) as minor components, form fibrils [41]. Although the major component from bovine lens crude protein extracts is α-crystallin, the exact protein composition of fibrils formed from this mixture remains to be explored, and could include incorporation of some β– and γ-crystallin subunits within the fibrils [41], both of which form fibrils on their own *in vitro* [38, 42, 43]. Recently, the highly-ordered nature of amyloid fibrils has generated significant research interest within the nanotechnology industry as self-assembling nanofibres, with potentially wide ranging applications as

gelation agents, viscosifiers, nanowires and scaffolds for cellular support and for enzyme immobilisation [44-46]. Crude bovine crystallin protein extracts represent an industrial waste product from the meat industry. It has been suggested that the ability to form fibrils from this crude mixture, without the need for time-consuming and expensive purification procedures, could be advantageous in the large-scale production of fibrils as bionanomaterials [41].

3.1 Solution conditions that induce α-crystallin amyloid fibril assembly in vitro.

Fibril formation by α-crystallin is favoured when it is subjected to partially denaturing conditions and incubation at elevated temperature, i.e. sufficient destabilization of the native state is required to give the protein the opportunity to rearrange and self-assemble into amyloid fibrils [38], as has been observed in other amyloid-forming systems [47, 48]. For example, bovine α-crystallin (as isolated from the lens and containing αA- and αB-crystallin subunits) forms amyloid fibrils when incubated at elevated temperature (60 °C) with 10 % (v/v) trifluoroethanol at acidic pH or with 1 M guanidine hydrochloride at neutral pH (Figure 4A; lower concentrations of guanidine hydrochloride were found insufficient to induce amyloid formation within 24 hours) [38]. Real-time 1D ^{1}H NMR spectroscopy was employed to monitor the time course for fibril formation by αB-crystallin and confirmed that major unfolding of the native protein precedes self-assembly into fibrils [39]. The ^{1}H NMR spectrum of the native αB-crystallin oligomer is well-resolved and arises solely from a solvent-exposed, flexible and unstructured 12-residue C-terminal extension. Resonances from the remaining part of the protein are not observed by ^{1}H NMR spectroscopy because the large native aggregate (mass of ~600 kDa) tumbles slowly [49, 50]. During amyloid fibril assembly by αB-crystallin, however, ^{1}H NMR resonances in the aromatic region of the spectrum became visible for a brief period during the early stages of aggregation [39]. Given that all the aromatic residues for αB-crystallin are located in the N-terminal and central (α-crystallin) domains, the implication is that these regions partially unfold prior to fibril formation [39].

3.2 Fibril formation by peptides derived from αA-crystallins.

Cross-linking studies have identified regions of αA-crystallin responsible for binding to proteins undergoing amorphous aggregation [51]. Synthesized peptides corresponding to these regions inhibit both amorphous target protein aggregation [51] and amyloid fibril formation [40]. For example, residues 71-88, (αAC(71-88)), corresponding to a putative chaperone target protein binding site, inhibit amyloid fibril formation by Aβ(1-40) [40]. Interestingly, αAC(71-88) itself assembles into amyloid fibrils *in vitro* at pH 7.5 and 37°C [40]. The ability of this αA-crystallin peptide to form amyloid fibrils is, however, suppressed when it is mixed with Aβ(1-40), suggesting that the chaperone

activity and the propensity to form amyloid fibrils may be closely linked. The link between chaperone action and amyloid fibril formation is further supported by the observation that residue F71 in αA-crystallin is important for both properties; removal of F71 from αAC(71-88) stifled both of these activities [40].

The close links between these apparently opposing behaviours, i.e. the ability to inhibit aggregation and also to self-aggregate, may be rationalized when the mechanism of sHsp chaperone activity is taken into account. The aptitude of sHsps to be effective chaperones arises, at least in part, from their sequences containing hydrophobic regions, such as amino acids 71-88 in αA-crystallin, that facilitate their interaction with exposed hydrophobic patches on partially unfolded target proteins to prevent their aggregation [52]. Whereas on one hand this property is useful for chaperone activity, on the other hand it may have detrimental consequences, since hydrophobic sequences also have a high propensity to aggregate and are prone to forming amyloid fibrils [10], as was observed for αAC(71-88) [40]. Full-length sHsp sequences may have evolved to counteract this issue, for example by flanking hydrophobic sequences with residues likely to suppress aggregation. This could include charged residues or flexible hydrophilic regions, that are likely to hinder aggregation [11, 12, 53]. Indeed, αAC(70-88), which has an extra lysine residue at the N-terminus, did not form fibrils [40], indicating that the charged K70 residue may play an important role in suppressing amyloid fibril formation in full-length αA-crystallin. The hydrophilic and highly flexible 10-12 amino acid C-terminal extensions of αA- and αB-crystallin [49, 50] may also play a role in hindering amyloid fibril formation by these proteins since they are likely to discourage formation of inter-molecular contacts that could lead to fibril formation [53].

3.3 Structural properties of fibrils formed from α-crystallins.

α-Crystallin forms fibrils with a variety of morphologies, depending on the experimental conditions employed. For example, the level of purity of α-crystallin, prepared from bovine eye lenses, was highly influential on the resultant fibril morphology as observed by TEM; short and curly fibrils were formed from crude crystallin protein extracts, and long, straight fibrils arose from purified bovine α-crystallin [41]. Imaging studies of human recombinant αB-crystallin fibrils reveal unusual and apparently branched morphologies at physiological pH in the presence of guanidine hydrochloride (Figure 4E) [39]. These ‘mature’ fibrils, comprising multiple protofilament assemblies, were found to unravel when the pH of solution containing the fibrils was altered (Figure 4E-G) [39]. For example, changing the pH from 7 to 2 makes the overall charge of αB-crystallin more positive and leads to significant repulsion between molecules, shifting the equilibrium between the protofilament

assemblies and resulting in dissociation and large morphological changes (as assessed by AFM, Figure 4E-G) [39]. The dissociated fibrils at acidic pH displayed 'protofibrillar' morphologies and possessed a relatively low elastic modulus, weak intermolecular interactions, and less regular structures in comparison to a range of 'mature' amyloid fibrils formed from a variety of proteins (Figure 4F) [24]. These flexible protofibrillar morphologies readily assembled into amyloid pores (Figure 4G), similar to those reported for other amyloid systems, e.g. apolipoprotein C-II [54, 55] and α-synuclein [56]. Interestingly, this morphology has been proposed to represent a toxic species [56, 57].

Overall, a combination of biophysical studies, including NMR spectroscopy and AFM imaging, has revealed important insights into the structural properties of α-crystallin amyloid fibrils formed *in vitro*. For example, ^{1}H NMR studies of αB-crystallin amyloid fibrils show that the flexible C-terminal is not incorporated into the structured cross-β sheet fibrillar core, but instead protrudes into solution [39], i.e. the extension is flexible in both the fibrillar [39] and native αB-crystallin aggregate [49]. The C-terminal extension plays an important role in maintaining solubility of the native protein and the oligomeric complexes it forms with target proteins in preventing their aggregation [49, 50, 58], and therefore may also influence the solubility of the αB-crystallin fibrils.

4. Amyloid fibril formation by the small heat-shock proteins: a disease role *in vivo*?

Amyloid fibril formation by sHsps may be linked to disease formation, in particular lens cataract and desmin-related cardiomyopathy (DRM). The following sections discuss these associations in detail.

4.1 Amyloid fibril formation in the eye lens: cataract and α-crystallin

Cataract is defined as opacity of the eye lens. In the healthy eye lens, transparency is thought to be maintained by a liquid-like, short-range order that is present in highly concentrated solutions of the crystallins [59]. The crystallins are organised in a stable supramolecular β-sheet structure within the healthy eye lens and α-crystallin plays an important role in chaperoning the other lens crystallins (β and γ), by suppressing aberrant aggregation that would otherwise cause light scattering and impair vision [2]. It has been generally considered that the opacity present in cataractous lenses arises from amorphous aggregation of the crystallin proteins. There is increasing evidence to suggest, however, that amyloid fibril formation by crystallin proteins within the lens could be the cause of certain forms of cataract. For example, in a murine model for cataract, a direct causal relationship was reported between the disease state and amyloid fibril formation by a natural deletion mutant from γ-crystallin [43]. Additionally, a connection has been reported between AD and supranuclear cataract [60]. *Ex vivo* investigation of lenses from individuals with AD revealed the accumulation of Aβ peptides

colocalised with αB-crystallin in electron dense deposits that exhibited birefringence with Congo Red staining and a positive interaction with thioflavin S, both of which bind strongly to amyloid fibrils [60]. These electron dense deposits correlated with the same areas of the lens where cataract was identified by slit lamp examination. *In vitro*, Goldstein *et al.* [60] demonstrated that Aβ bound αB-crystallin with high affinity, promoting the formation of aggregates and amyloid protofibrils. Overall, it was suggested that the presence in the lens of Aβ may promote protein aggregation, amyloid fibril formation and supranuclear cataract [60].

There is no direct *in vivo* evidence, however, to show that fibril formation by α-crystallin occurs within the eye lens. Nevertheless, intuitively the lens environment may be favourable to amyloid fibril assembly. For instance, the concentration of α-crystallin is high (approximately 150 mg/ml) and there is very little protein turnover within the avascular lens and it contains proteins as old as the individual [2]. With time, there is a mounting strain of longevity exerted on the crystallins as they degrade and undergo an increasing level of post-transitional modifications in old age (e.g. truncation, phosphorylation, glycation, deamidation) that could lead to destabilization of their native state [61]. Furthermore, crystallin protein aggregation occurs slowly. As a result of these factors, an opportunity may arise for the crystallins to rearrange and self-assemble into amyloid fibrils. Furthermore, Frederikse reported that healthy mammalian eye lenses stain positively with the amyloidophilic dyes Congo red and Thioflavin [62], implying that crystallins in the lens reside in a 'amyloid-like' β-sheet supramolecular structure [62], and thus it is conceivable that the conformational change required to form amyloid fibrils could be relatively minor. Additionally, as summarized above, the sHsp α-crystallin readily forms amyloid fibrils *in vitro* when subjected to partially destabilizing conditions [38].

Further work is required, however, to determine whether many forms of cataract can be attributed to amyloid fibril aggregation, and additionally the role α-crystallin may play in this process, i.e. whether it suppresses amyloid assembly by the other lens crystallins (i.e. β- and γ-crystallin), and/or whether it forms amyloid fibrils itself in the eye lens.

4.2 Amyloid fibril formation by R120G αB-crystallin leads to desmin-related cardiomyopathy (DRM).

The naturally occurring missense mutant R120G αB-crystallin causes desmin-related cardiomyopathy (DRM), an autosomal dominant disorder presenting in late adulthood with symptoms of cardiomyopathy, cataract, and desmin aggregation in muscles [63, 64]. The exact nature and causes of

the pathogenicity involved in R120G αB-crystallin-induced cardiomyopathy remain to be fully understood, although reductive stress is likely to play an important role [65]. Parallels have been drawn, however, between DRM and neurodegenerative diseases such as AD [66], and the pathogenic processes associated with DRM may additionally involve the formation of toxic amyloid oligomers by R120G αB-crystallin [67]. Electron dense deposits have been identified containing R120G αB-crystallin which stain positively with the dye Congo red and are also immunoreactive with conformationally dependent epitope antibodies that recognize amyloid oligomers [67]. In a cardiac-specific transgenic mouse model of this disease, R120G αB-crystallin amyloid oligomer formation produced mitochondrial dysfunction and apoptosis [68]. Terminating R120G αB-crystallin expression resulted in a decrease in amyloid oligomer formation and rescued the transgenic animals from premature death [69]. Prolonged voluntary exercise of the mice was also shown to reduce amyloid oligomer levels and slow the progression to heart failure [66].

In comparison to the wild-type protein, R120G αB-crystallin has a significantly destabilized structure that has the tendency to aggregate [70]. It readily forms amyloid fibrils *in vitro*, and possesses structural properties akin to fibrils formed from the wild-type protein, including an apparently identical morphology (as visualized by TEM) and a similar cross-β X-ray fibre diffraction pattern [39]. Amyloid formation by R120G αB-crystallin occurs with a slower growth rate than the wild-type protein *in vitro*. Given early amyloid oligomers are toxic to cells [20], the ability of R120G αB-crystallin to persist in this form was suggested to be consistent with its potentially pathogenic role *in vivo* [39].

Sanbe *et al.* [71] showed that recombinant R120G αB-crystallin forms toxic amyloid oligomers *in vitro*, approximately 240-480 kDa in mass. The addition of other sHsps, Hsp22 and Hsp25, blocked oligomer formation by R120G αB-crystallin and recovered cell viability in a cardiomyocyte-based based study, whereas wild-type αB-crystallin enhanced cell toxicity, suggesting that the mutant and wild-type αB-crystallin co-aggregate as toxic oligomers [71]. Thus, one therapeutic approach in the treatment of DRM may involve elevating cellular levels of Hsp22 and Hsp25.

5. sHsps are associated with amyloid plaques and deposits in protein conformational diseases.

Apart from their ability to form amyloid fibrils when placed under destabilising conditions (see above), there is a growing body of evidence to show that the chaperone activity of sHsps prevents other proteins from forming amyloid fibrils and that sHsps are intimately associated with protein conformational diseases [37]. For example, sHsps have been found within amyloid fibril deposits and plaques [72-76] and the expression of some sHsps is dramatically up-regulated in response to

pathological conditions associated with fibril formation, e.g. in Dementia with Lewy bodies [75, 77], Alexander's disease [78-80], Creutzfeldt–Jakob disease [72], AD [73, 81] and other neurological conditions [82, 83]. The up-regulation of sHsps expression is presumably a stress response, at the cellular level, to the aggregating species and is thought to reflect an attempt by the cell to mitigate fibril formation.

It remains to be established what effect sHsps have on the toxicity associated with fibril formation. In transgenic *Caenorhabditis elegans* worms, the expression of human Aβ(1-42) led to the induction of Hsp16 proteins (which are homologs of αB-crystallin in vertebrates) [84]. Moreover, the Hsp16 proteins co-localized and co-immunoprecipitated with Aβ(1-42) in this model [84] and increasing the expression of Hsp16 partially suppressed the Aβ-mediated toxicity to these worms [85]. Similarly, the toxicity associated with ataxin-3 aggregation in a *Drosophila* model of spinocerebellar ataxia type-3 (a poly-glutamine mediated protein conformational disease) was reduced when αB-crystallin was over-expressed in this system [86]. Cell-culture models of α-synuclein aggregation have shown that the mammalian sHsp, Hsp27, inhibits both the aggregation and toxicity associated with this process [77, 87]. It was concluded from both the Aβ and α-synuclein studies that sHsps inhibit fibril formation by acting early during the aggregation process to prevent the formation of toxic species [85] [77, 87]. This finding is supported by *in vitro* studies into the ability of sHsps to inhibit amyloid fibril formation (see below).

A possible mechanism by which sHsps may prevent cytotoxicity associated with fibril formation is suggested by studies showing that Hsp27 and αB-crystallin increase the resistance of cultured cells [88-90] and mice [65, 91] to oxidative stress. This may be significant with regard to amyloid fibril formation since it has been reported that the toxicity of prefibrillar amyloid species is due to the production of reactive oxygen species by the aggregating fibril-forming protein, which are generated as a consequence of fibril formation [92]. Therefore, sHsps may protect cells from the cytotoxic effects of fibril formation both through their ability to inhibit the process directly and to mediate the redox resistance of cells.

5.1 sHsps inhibit amyloid fibril formation in vitro.

Despite the up-regulation of sHsp expression and their co-localization with amyloid fibril deposits in various protein conformational diseases, the precise role of sHsps in interacting with the aggregating, fibril-forming target protein remains to be established [37]. The *in vitro* inhibition of fibril formation by sHsps of a variety of target proteins has now been demonstrated. For example, αB-crystallin, the most studied sHsp in this context, inhibits the fibrillation of α-synuclein [93-95], β2-microglobulin

[96] (Esposito, Carver, *et al.*, unpublished results), κ-casein [97, 98], insulin [99], the synthetic ccβ-Trp peptide [97], apolipoprotein C-II [100], and the prion protein (Ecroyd, unpublished results). Whilst there has been some conjecture over whether sHsps are able to inhibit fibril formation by the Aβ peptides [96, 101-103], our recent results suggest that αB-crystallin is a potent inhibitor of fibril formation by Aβ1-40 and Aβ1-42 and, in doing so also prevents the cytotoxicity associated with this process (Dehle, Ecroyd, Carver, unpublished results). Thus, along with the well established activity of sHsps to inhibit the disordered (amorphous) forms of protein aggregation, it is evident that sHsps also have a generic ability to inhibit ordered amyloid fibril protein aggregation. However, the mechanism(s) by which sHsps inhibit fibril formation appear to vary, not only from the one(s) utilised to inhibit amorphous aggregation, but also between different fibril-forming target proteins and under various solution conditions [97].

5.2 Mechanisms by which sHsps inhibit amyloid fibril formation.

αB-Crystallin acts at very low sub-stoichiometric ratios to prevent fibril formation by apolipoprotein C-II and does so by forming a transient complex with the partially folded form of the protein, which stabilizes it and enables it to refold back to its native state via the reversible on-folding pathway [100]. In contrast, when αB-crystallin prevents fibril formation by α-synuclein [93, 94] and κ-casein [97], it does so by forming a stable, soluble chaperone-target protein complex with the target protein (i.e. a 'reservoir of intermediates') in a manner presumed to be analogous to the mechanism of chaperone action used by sHsps against amorphously aggregating proteins [9]. In addition, in the case of α-synuclein, αB-crystallin redirects the protein from the amyloid fibril-forming pathway to the amorphous aggregation pathway [93], a process that is similar to the mechanism by which the polyphenol (-)-epigallocatechin-3-gallate (EGCG) inhibits fibril formation [104]. Redirecting the aggregation of a target protein from an ordered (amyloid fibril) to disordered (amorphous) pathway would benefit the cell since amorphous aggregates are typically non-toxic and more easily degraded via the ubiquitin-proteasome system.

There are also mechanistic differences in the manner by which sHsps interact with and inhibit protein aggregation leading to amyloid fibril formation as compared to amorphous aggregation. This conclusion is based on studies which have compared the chaperone activity of mutant and/or chimeric forms of sHsps against amorphous and amyloid fibril-forming target proteins. For example, Raman et al. (2005) [96], using chimeric α-crystallin proteins, in which the N-terminal domain of αA-crystallin was fused with the C-terminal domain of αB-crystallin (αANBC) and vice-versa (αBNAC), showed that whilst αANBC has no ability to prevent the reduction-induced amorphous aggregation of insulin

B-chain [105], it was as effective as wild-type αA-crystallin in inhibiting amyloid fibril formation by Aβ(1-40) and β2-microglobulin [96]. Similarly, the chaperone activity of the R120G αB-crystallin mutant (which, as discussed above, is linked to DRM and early onset cataract [64, 106, 107]) towards amorphously aggregating target proteins is significantly reduced compared to the wild-type protein [70, 108], however, its ability to prevent fibril formation by Aβ(1-40) is only slightly reduced [96]. A double mutant (E164A/E165A αB-crystallin) in the highly flexible and unstructured C-terminal extension of αB-crystallin [6, 9, 50, 58], had a markedly reduced ability to inhibit the reduction-induced amorphous aggregation of insulin and heat-induced amorphous aggregation of β_L-crystallin, but significantly increased capacity to prevent fibril formation by κ-casein and ccβ-Trp [109]. Together, these results indicate that there are distinct differences in the mechanism by which sHsps inhibit these two modes of protein aggregation. Interestingly, this raises the possibility that mutant forms of sHsps may be designed as more effective inhibitors of fibril formation compared to the wild-type protein and therefore may be an avenue for therapeutic potential in the future [109].

Recent work has shown that sHsps interact directly with amyloid fibrils by binding along their length, inhibiting their further aggregation and ability to 'seed' the formation of more fibrils [96, 99] (unpublished data). αB-Crystallin does not, however, have the ability to disassemble preformed fibrils [94]. The binding of αB-crystallin to fibrils is intriguing, as such a process may help to explain why sHsps are associated with amyloid deposits and plaques in protein conformational diseases (see above). Moreover, our recent results suggest that the interaction of αB-crystallin with preformed fibrils may result in them tangling and therefore the interaction of Hsps with fibrils may expedite plaque formation *in vivo*. Further studies in this exciting new area of sHsp-fibril research will hopefully shed more light on this interaction and the consequence for plaque progression in diseases associated with fibril formation.

6. Summary

Overall, a range of studies have demonstrated that the sHsp α-crystallin readily assembles into amyloid fibrils *in vitro* when subjected to partially destabilizing conditions. The potential role of amyloid fibril formation by α-crystallin in the bio-nanomaterial industry, and also in diseases such as cataract and DRM has been discussed. Whilst amyloid fibril assembly by other sHsps has not been reported to date, given that the central α-crystallin domain (of approximately 90 amino acids in length) is conserved between all sHsps and is likely to adopt a β-sandwich structure, these proteins may also have the potential to form amyloid fibrils. Additionally, there may be a close relationship between sHsp chaperone action and the propensity to self-aggregate and form fibrils, since both these activities

are favoured by exposed stretches of hydrophobicity. It is interesting that α-crystallin (along with other crystallins) adopts an amyloid-like ordered structure within the healthy eye lens. Overall, therefore, there may be a delicate balance between the structure and chaperone function of the native α-crystallin oligomer and the formation of amyloid fibrils. With regard to their ability to inhibit protein aggregation, including amyloid fibril formation, sHsps have significant therapeutic potential [83, 110, 111]. For example, sHsps could be used directly to suppress protein aggregation whereby enhancing their expression and/or chaperone ability chemically or by mutagenesis would have therapeutic application in the treatment of cataract and other protein misfolding diseases.

7. Acknowledgements

We thank Thomas P.J. Knowles (University of Cambridge) for acquisition and analysis of the AFM images shown in Figure 3. Our work described in this chapter has been supported by grants from the National Health and Medical Research Council (NHMRC) of Australia and Australian Research Council. HE was supported by a NHMRC Peter Doherty Fellowship and SM is supported by a Royal Society Dorothy Hodgkin Fellowship.

8. Abbreviations

Aβ; amyloid-beta, AD; Alzheimer's disease, AFM; atomic force microscopy, DRM; desmin-related cardiomyopathy, sHsp; small heat-shock protein, TEM; transmission electron microscopy, ThT; thioflavin T

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10. Figure legends

Figure 1. Amyloid fibrils arise from the association of a partially structured intermediate that deviates from the protein folding pathway. In this pathway, proteins move from their unfolded to folded/native states via an intermediate or intermediates. Amorphous aggregates arise from these states via relatively rapid association that leads to disordered precipitates whereas the formation of fibrils occurs over a longer timeframe via a nucleation and ordered growth mechanism involving a soluble precursor.

Figure 2. Schematic of sHsp chaperone action with amorphously aggregating proteins [6, 112]. Along the rapid folding pathway, the intermediately folded states of proteins are transiently present. The off-folding pathway is slow by comparison. As a result, the more disordered intermediate or molten globule (M.G.) state of the target protein (I_2), which is highly dynamic and displays significant hydrophobicity to solution, is present for a significant period of time and therefore has the propensity to aggregate along the off-folding pathway via a nucleation-dependent mechanism. sHsps exist as large, dynamic aggregates which are in constant exchange with dissociated, probably dimeric species. The dissociated sHsp may be more chaperone-active than the aggregated species, potentially via exposure of its chaperone binding site region during subunit dissociation. The aggregation of I_2 is also a dynamic process involving equilibria between various aggregated species. The dynamic nature of both protein species may be the trigger that facilitates sequestration of I_2 by the sHsp dimer. In support of this, sHsps specifically interact with target proteins that are aggregating via a nucleation-dependent mechanism [113]. Following binding to I_2, the complex is incorporated into a high-molecular mass aggregate. Refolding of I_2 to the native state (N) can occur via the action of another chaperone protein, e.g. Hsp70, which requires the hydrolysis of ATP. The squiggly lines on the surface of the sHsp oligomer and dimer represent the highly mobile and unstructured region of the C-terminal extensions [6, 49, 50, 58].

Figure 3. Structural model of an amyloid fibril, formed by the SH3 domain derived from cryo-EM and X-ray fibre diffraction data (reproduced from [29]). An amyloid fibril is a highly structured arrangement of the polypeptide chain that is characterised by an array of stacked cross β-sheets (b,d). The individual β-strands are oriented perpendicular to the long axis of the fibril (b,d) and stack to form protofilaments (b). The fibril may comprise more than one twisted protofilament, i.e. four in this case (a,c,d) which helically assemble into the overall fibril structure (a). Fibrils can be very long (hundreds of nm in length) (c), and may be highly stable and insoluble when sufficiently large (Fig. 1). The amyloid fibril is proposed to be a generic structure that is accessible to all polypeptide chains irrespective of their sequence [3].

Figure 4. A-D, Transmission electron microscopy (TEM) images of amyloid fibrils formed from A, bovine α-crystallin (purified); B, human recombinant αA-crystallin; C, human recombinant αB-crystallin; and D, human recombinant R120G αB-crystallin. Amyloid fibrils were formed by dissolving the protein at 1 mg/ml in the presence of 1 M guanidine hydrochloride (GdnHCl) (pH 7.4) and incubating at 60 °C for 2-24 h. E-G, Atomic force microscopy (AFM) images of human recombinant αB-crystallin fibril solutions prepared at pH 7.4 with GdnHCl, then, following a ten-fold (F), or one 100-fold (G), dilution into a pH 2.0 solution. In A-D, the scale bars represent 200 nm and in E-G, the scale bars represent 500 nm.

Table 1. Summary of *in vitro* studies of amyloid fibril formation by α-crystallins.

sHsps	**Protein Source**	**Ref.**
αT-Crystallin, mixture of αA + αB-crystallin	Bovine lenses (purified)	[38]
αT-Crystallin, mixture of αA + αB-crystallin	Bovine lenses (crude extracts)	[41]
Wild-type αA-crystallin, 173 aa	Human recombinant	[39]
αA-Crystallin, range of peptide fragments	Human recombinant	[40]
Wild-type αB-crystallin, 175 aa	Human recombinant	[39]
R120G αB-crystallin, 175 aa	Human recombinant	[39]

Figure 1

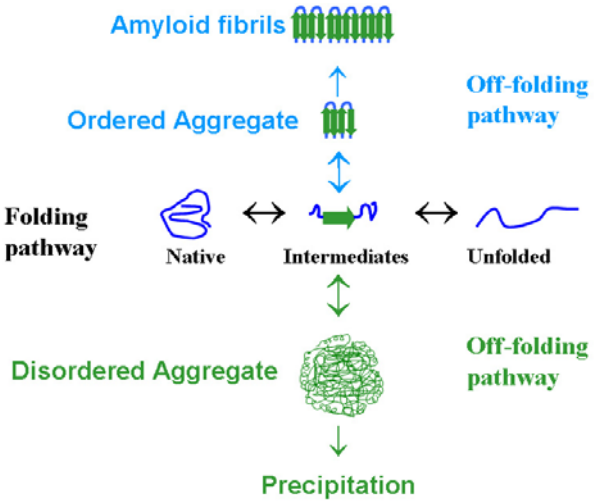
Amyloid fibrils
Ordered Aggregate
Off-folding pathway
Folding pathway
Native
Intermediates
Unfolded
Disordered Aggregate
Off-folding pathway
Precipitation

Figure 2

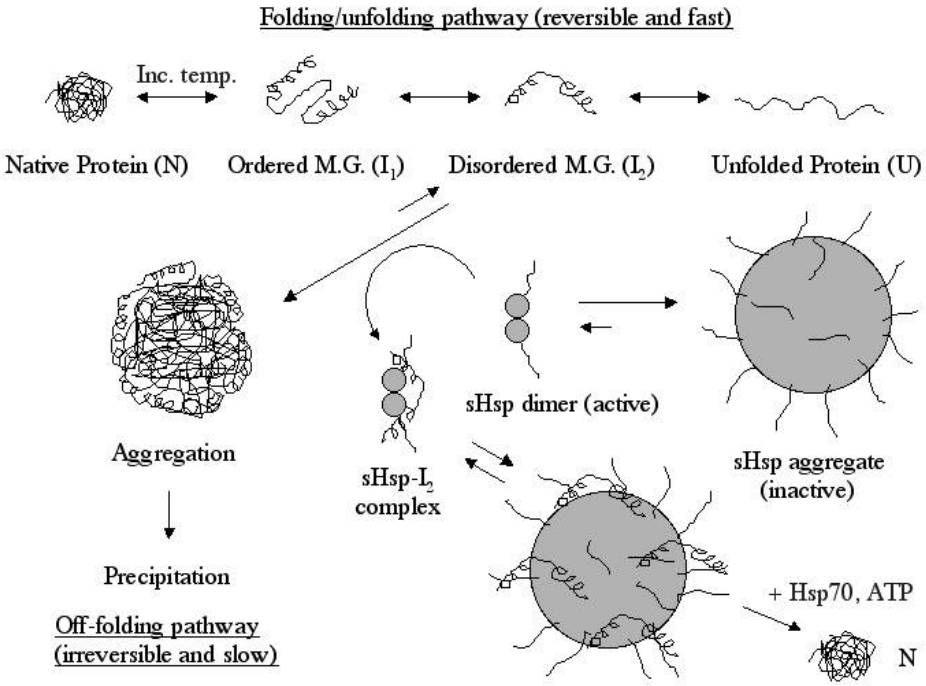
Folding/unfolding pathway (reversible and fast)
Inc. temp.
Native Protein (N)
Ordered M.G. (I1)
Disordered M.G. (I2)
Unfolded Protein (U)
Aggregation
Precipitation
Off-folding pathway
(irreversible and slow)
sHsp-I2
complex
sHsp dimer (active)
sHsp aggregate
(inactive)
+ Hsp70, ATP
N

Figure 3

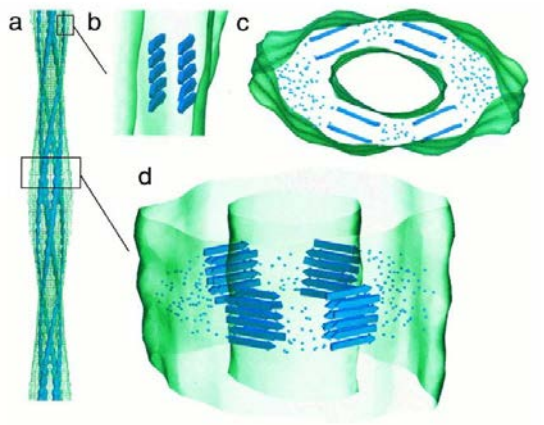

Figure 4